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Respected Sir,

Sub: Submission of final-synopsis

Ref: MU/EXB/Ph.D./CR.06/Chem./2022-23/E.25

Inviting reference to the above, I am submitting the final- synopsis of my Ph.D. thesis entitled "**Preparation and Characterization of Functionalized halloysite nanocomposites for Bio-medical applications**" for the degree of Doctor of Philosophy in Chemistry. I request you to kindly do the needful and oblige.

Thank you,

yours faithfully,

(**Deepali Warale**)

Forwarded with the recommendation to the Chairperson, Department of Chemistry, Mangalore University for onward transmission to the Registrar (Evaluation), Mangalore University.

Date: 04/06/2024
Place: Mangalagangothri

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# PREPARATION AND CHARACTERIZATION OF FUNCTIONALIZED HALLOYSITE NANOCOMPOSITES FOR BIO-MEDICAL APPLICATIONS

**FINAL SYNOPSIS**

**OF THE Ph.D. THESIS TO BE SUBMITTED**

**TO**

**MANGALORE UNIVERSITY**

**FOR THE AWARD OF DEGREE OF**

**DOCTOR OF PHILOSOPHY**

**IN**

**CHEMISTRY**

**BY**

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**Title of the thesis : Preparation and Characterization of Functionalized Halloysite Nano-Composites for Bio-Medical Applications**

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## Introduction

Over the course of the previous several decades, biomaterials have been used to advance biological applications. Applying surface modification techniques to biomaterials can change their dynamic properties, which can impact different biological processes in a specific biomedical setting [1]. Biomaterials derived from natural or synthetic sources possess inherent features, such as the capacity to respond to cell growth and undergo biodegradation, which confer them with superior advantages compared to their counterparts [2]. The investigation of cellular behaviour in biomaterials, including but not limited to cell proliferation, cell adhesion, and cytocompatibility properties, is of utmost importance [3,4]. Extensive research efforts have been dedicated to the investigation of biomaterials with the primary objectives of facilitating wound healing processes and advancing the field of tissue engineering. The production of films and scaffolds with specific architectural characteristics can be achieved by the combination of different materials and the generation of porous structures. Furthermore, it is possible to alter the thermo-mechanical properties of both the films and scaffolds [5,6]. The fabrication of scaffolds for wound healing and tissue engineering commonly involves the utilisation of naturally occurring biopolymers such as chitosan, sodium alginate, silk, cellulose, collagen, and related materials. When the integration of natural biopolymers with synthetic biopolymers occurs, commonly referred to as "bio mimicking," it enables a more efficient approach to biological research with a specific focus on the development of specialised applications [7].

Nanocomposite films and scaffolds composed of both natural and synthetic polymers have emerged as a prominent area of research interest [8]. These innovative materials have attracted the attention of researchers aiming to enhance the properties of manufactured goods by manipulating nanofillers, which possess at least one dimension smaller than 100 nm. Nanocomposites have garnered considerable attention in the biomedical domain because of their environmentally friendly nature, high efficiency, and renewable characteristics[9]. The manufacture of nanocomposites with improved thermo-mechanical properties, suitable for tissue regeneration and wound healing, often involves including nanofillers such as nanospheres, nanotubes, nanoplatelets, and similar materials into the polymer matrix. The incorporation of nanoclay components into the biopolymer blend enhances the mechanical strength and promotes improved cell adhesion and proliferation in scaffolds used for tissue engineering and wound healing [10–12].

Biopolymers such as lignin, sodium alginate, and gelatin are extensively employed in the domain of scaffold production for tissue regeneration. Lignin is the phenolic polymer that is most widely present in abundance. The interaction between lignin and hemicellulose produces ether or ester linkages [13]. Lignin's complex cross-linking structure is due in part to the fact that it has different functional groups, like aliphatic, phenolic, and methoxyl. The reactivity and chemical characteristics of polymers are influenced by the hydroxyl groups and aromatic structure of lignin [14,15]. The hydroxyl group that is most commonly seen in this compound is aliphatic in nature. The quantities of hydroxyl groups exhibit variation depending on the source of lignin [16]. The suitability of lignin films for medicinal applications was assessed. The field of tissue engineering and regenerative medicine

biomaterials encompasses a wide range of academic fields [17]. Scaffolds are subject to several regulatory mechanisms, some of which may exhibit conflicting effects based on their specific functional roles. Within the field of orthopaedic surgery, there is a significant focus on drug distribution and wound therapy. Sodium alginate is a naturally occurring polysaccharide composed of α-L-guluronic acid (M) and β-D-mannuronic acid (G). The substance is derived from the cellular structure known as the cell wall of brown algae [18]. Sodium alginates possess advantageous characteristics such as biocompatibility, reduced toxicity, and affordability. Sodium alginate possesses the potential for modification through many methodologies, making it suitable for a range of biological applications, including wound healing, drug administration, tissue regeneration, and similar purposes. Alginates have shown considerable potential as biomaterials for facilitating tissue regeneration by their ability to enhance cell proliferation, promote cell adhesion, and facilitate cellular transplantation [19,20]. Gelatin, a proteinaceous material derived from the hydrolysis of collagen, has been extensively used in several biological domains. Gelatine is a protein composite that originates from collagen and consists of the same amino acid sequences[21]. Collagen has higher tertiary structure than gelatine, which makes it less aqueous soluble. Different types of animal collagens may be used to create gelatine, with porcine, fish, and bovine collagens being the most popular for use in tissue engineering[22]. Because of its many useful properties, including being biocompatible, biodegradable, non-immunogenic, cell-interactive, and commercially available, gelatine is frequently employed in biomedical applications. Because of its high biocompatibility, cheap processing cost, and wide availability, gelatine has been utilised for years in pharmaceutical formulation, cell culture, and tissue engineering. In addition, the polymer structure of gelatin includes an arginine-glycine-asparagine (RGD)-like sequence, which promotes cell adhesion and motility[23].

Halloysite nanotubes (HNTs) ($Al_2Si_2O_5(OH)_4.nH_2O$) are a naturally occurring aluminosilicate nanoclay biomaterial that can be taken from natural deposits and amorphous allophones that have formed over a long period of time [24]. The Northland (New Zealand) and Dragon Mine (United States) deposits contain the most HNTs (in thousand tonnes of purified product). HNTs are environmentally compatible, non-toxic, and economical. Depending on their origin, HNTs can take various forms, including short tubes, large tubes, and spheroids [25]. HNTs are cylindrical tubes with an outer surface composed of siloxane (Si-O-Si) groups, an inner surface and margins composed of Al-OH groups with lengths ranging from 200 to 2000 nm, and an outer and interior diameter of 50–70 nm and 10–20 nm, respectively [26,27]. Nanomaterials, which typically have dimensions ranging from 1 to 100 nm, have garnered significant attention in recent decades because of their extensive utilisation in several fields such as healthcare, energy storage, catalysis, and sensing [8]. Silver nanoparticles (AgNPs) have garnered significant interest, particularly within the field of biomedicine. Additional therapeutic uses of silver nanoparticles (AgNPs) encompass wound healing, bone regeneration, dental treatments, vaccine enhancement, anti-diabetic characteristics, and bio-sensing [9]. These applications complement the primary focus on the antibacterial and anticancer attributes of AgNPs, as well as their underlying processes. HNTs' outer and interior surfaces support selective modification, which facilitates uniform dispersion in polar polymers such as epoxy, polyamides, polyethyleneimine, polyvinyl alcohol, polyacrylates, and biopolymers such as polysaccharides such as pectin, lignin, gelatin, and alginate. HNTs also disseminate uniformly with medium- and low-polarity polymers such as polyvinylchloride, polyethylene, and polypropylene.

Specifically, the incorporation of lignin, alginate, and biogelatin on the outer surface of nanotubes (HNTs) has been found to enhance cell proliferation within porous scaffolds. Polyvinyl alcohol (PVA) is a synthetic biomaterial that finds use in the fields of tissue engineering and wound dressing. The polymer under consideration is characterised by its water solubility, lack of toxicity, and favourable attributes in terms of mechanical strength, flexibility, and film-forming capabilities. Polyvinyl alcohol (PVA) possesses a pronounced hydrophilic characteristic, resulting in diminished cell adhesion properties in PVA-based scaffolds or films. However, this drawback can be mitigated by including halloysite nanotubes (HNTs) into the PVA matrix, as suggested by previous studies [28–30].

The thesis showcases the modification of raw halloysite nanotubes (HNTs) using two distinct methods: surface roughening to create surface-roughened HNTs and embedding nanosilver onto HNTs to produce nanosilver embedded HNTs. The modified halloysite nanotubes (HNTs) were further subjected to grafting with the biopolymers lignin, sodium alginate, and gelatin. The nanocomposite film production involved the reinforcement of grafted nanotubes onto a matrix of polyvinyl alcohol. The surface roughening of pure halloysite nanotubes (HNTs) was achieved using a manual grinding procedure using a combination of sodium carbonate ($Na_2CO_3$) and sodium nitrite ($NaNO_2$), followed by the calcination of the resulting mixture. The resulting powder is employed for the grafting of lignin and sodium alginate. The lignin grafting procedure was conducted in a solution of 0.1M NaOH under continuous stirring similarly incorporating sodium alginate into distilled water. Consequently, the nanofillers were appropriately produced and fabricated into the water-soluble polymer known as polyvinyl alcohol (PVA) to create PVA/Lig/rHNT and PVA/Sod.alg/rHNT nanocomposite films. These films were fabricated using different concentrations of nanofillers. Another modification performed is the application of nanosilver onto the external surface of untreated HNTs. The aforementioned procedure involves the use of surface alkali etching for an extended duration, often overnight. Subsequently, the resulting dried product is subjected to treatment with $AgNO_3$ in a standardised proportion. The combination of these two substances was transformed into a slurry and subjected to calcination at a temperature of $450^0$C for a duration of one hour. The resulting product successfully underwent a washing and separation process, resulting in the predicted product, namely AgNp-HNTs. The resulting modified product was grafted with both lignin powder and gelatin powder. Novel nanofillers were synthesised and then used in the production of PVA nanocomposite films, namely in the formulations PVA/Lig/AgNp-HNT and PVA/Gel/AgNp-HNT.

A variety of instrumental techniques, including Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), thermal analysis (TGA and DSC), universal tensile machine (UTM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and water content angle (WCA), were used to characterise the fabricated nanocomposite films. The study examined the swelling behaviour of nanocomposite films at a pH of 7.4 in phosphate buffer saline. In the presence of lysozyme (egg albumen), *in-vitro* enzymatic degradation was carried out in phosphate buffer saline (pH 7.4). Human erythrocytes were used to perform hemolysis. For *in vitro* biocompatibility and cytocompatibility experiments, such as cell proliferation, adhesion, and migration, mouse fibroblast (NIH3T3) cell lines were used to investigate nanocomposite films. It is being investigated if nanocomposite films can be used in biological applications.

**Chapter 1:** General Introduction

**Chapter 2:** Materials and methods

**Chapter 3:** Surface alteration of the raw HNTs

- **3.1** Surface roughening of pure HNTs
- **3.2** Characterization of rHNTs.
- **3.3** Modification of HNTs into Silver embedded HNTs(AgNp-HNTs)
- **3.4** Characterization of AgNp-HNTs

**Chapter 4:** Functionalization & characterization of rHNTs and AgNp-HNTs with organosolv-lignin, Sodium alginate, and gelatin.

- **4.1** Functionalization of rHNTs as Lig/rHNTs
- **4.2** Functionalization of rHNTs as Sod.alg/rHNTs
- **4.3** Functionalization of AgNp-HNTs as Lig/AgNp-HNTs
- **4.4** Functionalization of AgNp-HNTs as Gel/AgNp-HNTs

**Chapter 5:** Fabrication of nanocomposite films by prepared nanofillers.

**5a:** Lignin functionalized rough halloysite nanotubes (rHNTs) incorporated poly (vinyl alcohol) (PVA) nanocomposites promote Cell proliferation and Cell adhesion.

- **5a.1**. Fabrication of Lig/rHNT/PVA nanocomposite films
- **5a.2**. Characterization
- **5a.3.** Swelling behavior, Mechanical traits and in-vitro enzymatic degradation
- **5a.4**. *In-vitro* cell line studies.

**5b:** Poly (vinyl alcohol)/sodium-alginate-functionalized rough-halloysite clay nanotube nanocomposites: potent biocompatible materials for wound healing applications

**5b.1.** Fabrication of Sod.alg/rHNT /PVA nanocomposite films

**5b.2.** Characterization

**5b.3.** Mechanical behavior and *In-vitro* enzymatic degradation

**5b.4.** Cytotoxicity and Scratch assay studies.

**5c:** Silver embedded halloysite nanotubes with lignin modification incorporated PVA bio-nanocomposite films: Thermal, mechanical properties and cell migration studies via wound closure rate.

**5c.1.** Fabrication of PVA/Lig/AgNp-HNT nanocomposite films.

**5c.2.** Characterization

**5c.3.** Thermal stability, Mechanical studies and *In-vitro* enzymatic degradation

**5c.4.** Cell-adhesion and cell-migration studies.

**5d:** *In-vitro* evaluation of nanosilver embedded halloysite nanotubes with biogelatin reinforced PVA/Gel/AgNp-HNT nanocomposite films for tissue regeneration and wound healing application.

**5d.1** Fabrication of PVA/Gel/AgNp-HNT nanocomposite films

**5d.2.** Characterization

**5d.3**. Thermal stability, Mechanical studies and *In-vitro* enzymatic degradation

**5d.4.** Bio-medical application.

**Chapter 1: General Introduction**

This chapter serves as an introduction, providing a comprehensive literature review on the surface modifications of pure halloysite nanotubes and the utilisation of biopolymers in the grafting process of these clay nanotubes. The focus of this study is on the reinforcement of nanofillers inside polymer matrices, namely those based on polyvinyl alcohol (PVA). The emphasis is placed on the incorporation of these nanofillers into nanocomposite films. The present study incorporates a literature review on the functionalization of halloysite nanotubes by the utilisation of lignin, sodium alginate, and

gelatin nanocomposite films, which are based on polyvinyl alcohol (PVA).

**Chapter 2: Materials and methods**

This chapter provides a comprehensive account of the materials and procedures employed in the functionalization of halloysite nanotubes (HNTs) and the subsequent construction of nanocomposite films. The methods utilised for the characterization of functionalized halloysite nanotubes (HNTs) and nanocomposite films have also been elucidated. The methodology utilised for conducting the swelling and in-vitro enzymatic degradation experiments has been delineated. The experimental methodology for studying biological processes such as cell proliferation, adhesion, and migration has been outlined. Additionally, this study incorporates the experimental methodology of hemolysis and the in-vitro assessment of anti-inflammatory effects.

**Chapter 3: Surface alteration of raw HNTs**

This chapter provides a detailed account of the surface modification of halloysite nanotubes using two distinct methods, referred to as sub-chapters 3.1 and 3.3. The first method involves roughening the surface of HNTs through a process of grinding with an inorganic salt mixture, followed by calcination. The second method involves the modification of HNTs into AgNp-HNTs by treating them with silver nitrate. The alterations were characterised using FTIR, PXRD, TGA, and FE-SEM methods. The findings are further discussed in sections 3.2 and 3.4.

**Chapters 4: Functionalization of biopolymers onto Surface altered HNTs**

This chapter focuses on the process of grafting lignin, sodium alginate, and gelatin molecules onto the surface of pre-modified HNTs. The chapter is further subdivided into sections 4.1, 4.2, 4.3, and 4.4. Each sub-chapter delineates the strategic approach employed in the process of functionalization. The outcomes derived from the analysis conducted using various measuring methodologies. The nanofillers derived from these sources include Lig/rHNTs, Sod.alg/rHNTs, Lig/AgNp-HNTs, and Gel/AgNp-HNTs.

**Chapters 5: Fabrication of PVA-nanocomposite films by prepared nanofillers.**

This chapter details the methodology employed to incorporate nanofillers into the chosen PVA matrix, with varying weight percentages. The chapter is divided into sub-sections 5a,

5b, 5c, and 5d, according to the various nanofillers used. The resulting films were then investigated, and the acquired results were documented.

### *5a: Lignin functionalized rough halloysite nanotubes (rHNTs) incorporated poly (vinyl alcohol) (PVA) nanocomposites promote Cell proliferation and Cell adhesion.*

This chapter deals with the fabrication of lignin-functionalized rHNTs into a PVA matrix to produce nanocomposite films via the solution casting technique. Using FTIR, PXRD, TGA, DSC, UTM, FESEM, AFM, and WCA, the nanocomposite films were thoroughly characterised. The swelling and in-vitro enzymatic degradation investigations were conducted with 7.4 pH phosphate buffer saline. The NIH3T3 mouse fibroblast cell line investigations, cell proliferation, and adhesion activities of the nanocomposite films have been presented. Using human erythrocytes, the hemocompatibility of the nanocomposite films has also been described. It has been investigated whether nanocomposite films can be utilised in tissue engineering applications. The outcome is discussed.

### *5b: Poly (vinyl alcohol)/sodium-alginate-functionalized rough-halloysite clay nanotube nanocomposites: potent biocompatible materials for wound healing applications.*

This chapter details the synthesis of PVA nanocomposites containing sodium alginate-functionalized roughened halloysite nanotubes. Films were fabricated using the solution casting technique. FTIR, PXRD, TGA, DSC, UTM, FE-SEM, AFM, and WCA techniques were utilised to analyse the prepared films. Using human red blood corpuscle cells, the hemolytic activity of nanocomposite films has been determined in vitro. Using mouse fibroblast NIH3T3 cells, in vitro cellular studies for nanocomposite films have been conducted to evaluate cell proliferation, cell adhesion, and cell migration activity. The nanocomposite coatings that were synthesised can be used effectively in wound healing applications. The outcome is discussed.

### *5c: Silver embedded halloysite nanotubes (AgNp-HNTS) with lignin modification incorporated PVA bio-nanocomposite films: Thermal, mechanical properties and cell migration studies via wound closure rate.*

This chapter describes the synthesis of PVA nanocomposites containing silver embedded halloysite nanotubes functionalized with lignin. Using the solution casting method, films were manufactured. The prepared films were analysed using FTIR, PXRD, TGA, UTM, FE-SEM, AFM, and WCA techniques. *In vitro* evaluation of the hemolytic activity of

nanocomposite films using human red blood corpuscle cells Using NIH3T3 mouse fibroblast cells, in vitro cellular studies for nanocomposite films were conducted to evaluate cell proliferation, cell adhesion, and cell migration activity. Synthesised nanocomposite coatings can be used effectively in wound healing applications. The results are discussed.

### 5*d: In-vitro evaluation of nanosilver embedded halloysite nanotubes with biogelatin reinforced PVA/Gel/AgNp-HNT nanocomposite films for tissue regeneration and wound healing application.*

The following section provides an account of the production process for polyvinyl alcohol (PVA) nanocomposites that have halloysite nanotubes coated with nanosilver, which have been modified with gelatin molecules. Films were produced utilising the solution casting technique. The films that were created underwent analysis with a range of techniques, including FTIR, TGA, UTM, FE-SEM, and WCA. The present study focuses on the *in vitro* assessment of the hemolytic activity exhibited by nanocomposite films by the use of human red blood corpuscle cells. Cellular investigations were done in vitro using NIH3T3 mouse fibroblast cells to assess the cell proliferation, cell adhesion, and cell migration activities of nanocomposite films. The utilisation of synthesised nanocomposite coatings has demonstrated efficacy in the context of wound healing applications. The findings are being analysed and deliberated upon.

**Summary and Conclusion**

The growing interest in biomaterial clay halloysite nanotubes and their numerous characteristics served as the inspiration for this study. A comprehensive study examined functionalized halloysite nanotubes in biocompatible and biodegradable nanocomposite polymers. The influence of functionalized HNT weight % on the characteristics of solution-cast nanocomposite films was examined. The initial stage of this research involved the surface alteration of halloysite nanotubes with alkali etching and surface coating with silver nanocparticles followed by the functionalization with biopolymers such as lignin, gelatin and sodium alginate. FTIR, TGA, PXRD, and FE-SEM corroborated this. The FTIR peaks of HNTs were not significantly altered during functionalization, confirming surface functionalization. Through PXRD and TGA research, we found that the functionalized HNTs' crystalline structure doesn't change their basic structure and that the composite material's thermal stability decreases. Overall, surface-functionalizing HNTs with lignin, gelatin and sodium alginate increases their bioactivity. The second phase of this research prepared functionalized HNTs using varying weight % reinforced in biocompatible

polymer like PVA with suitable biomedical qualities. The interaction between functionalized HNTs and polymer matrix improved the mechanical and thermal properties of nanocomposite films, increasing the weight percent from 1 to 5 wt%. According to PXRD results, the weight content of functionalized HNTs increased the crystallinity of nanocomposite films. FE-SEM-EDS and AFM topographic images showed that nanofillers were evenly spread out in nanocomposite films from 1% to 5% by weight, and the overall roughness got better. The roughness of the films may aid biological cell adhesion and proliferation. The hydrogen link between the matrix and filler lowers the ratio of nanocomposite films that swell, and the nanofiller makes the network denser, which also stops the films from swelling. Owing enzymatic breakdown of nanocomposite films is accelerated due to lysozyme interaction for glycoside bond cleavage. Further biocompatibility, hemocompatibility, and cytocompatibility experiments showed that nanocomposite films are non-toxic at all concentrations. Nanocomposite films made of PVA, all helped NIH3T3 mouse fibroblast cells grow, multiply, and stick together better at all concentrations. The studied nanocomposite films were cytocompatible because the cells had fibroblastic shapes. The blood compatibility assay showed that all PVA nanocomposite films were < 5% hemolytic, proving their biomedical suitability for tissue engineering. The scratch test of PVA nanocomposite films showed that they helped cells move into the wound more effectively than pure PVA film and hence these films could be used as wound healing materials. Due to their biocompatibility, cytocompatibility, and blood compatibility, nanocomposite films may be excellent for tissue engineering and wound healing.

## List of Publications

1. **Deepali Warale**, S. Kouser, G. K. Nagaraja, Shabeena M., D. J. Manasa. *In vitro* Cell proliferation, Adhesion Studies, and Enhancement of Mechanical properties of organo solve-Lignin functionalized Halloysite Clay Nanotube fillers doped onto Poly (Vinyl Alcohol) film. Surfaces and Interfaces (2022). doi.org/10.1016/j.surfin.2022.102593 **(Elsevier, I.F– 6.2)**

2. **Deepali Warale**, A. Prabhu, S. Kouser, G. K. Nagaraja, Shabeena M., D. J. Manasa Incorporation of Sodium Alginate Functionalized Halloysite nanofillers into Poly (Vinyl Alcohol) to Study Mechanical, Cyto/Heme compatibility and Wound Healing Application. International Journal of Biological Macromolecules (2023). doi.org/10.1016/j.ijbiomac.2023.123278 **(Elsevier, I.F – 8.2)**

3. **Deepali Warale,** Shabeena M., A. Prabhu, S. Kouser, G. K. Nagaraja, D. J. Manasa.

Sustainable organosolv-lignin coated Nanosilver- halloysites reinforced Poly (vinyl alcohol) nanocomposites for Wound healing application. International Journal of Biological Macromolecules (2023). doi.org/10.1016/j.ijbiomac.2023.128628 **(Elsevier, I.F- 8.2)**

## List of Papers under communication

1. **Deepali Warale**, Shabeena M., A. Prabhu, S. Kouser, G. K. Nagaraja, D. J. Manasa. Biogelatin-fouled Silver-Aluminosilicate clay nanotube supported Poly (vinyl alcohol) nanocomposites for Cell proliferation and Wound repair

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